PATENT
3631-0107P

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IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: JENSEN, MARTIN ROLAND ET AL. Conf.:

Appl. No.: 09/785,215 Group: 1647

Filed: February 20, 2001 Examiner: NICHOLS, C J

For: NOVEL METHOD FOR DOWN-REGULATION OF AMYLOID

DECLARATION SUBMITTED UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents May 13, 2003
Washington, DC 20231

Sir:

I, Peter Birk Rasmussen, of A.D. Jørgensensvej 31, DK-2000 Frederiksberg, Denmark, do hereby declare the following:

1. I am named co-inventor of the above-captioned patent application. I have attached a copy of my *curriculum vitae* to this Declaration.

2. I hold a PhD and am employed as Senior Scientist at Pharmexa A/S in Denmark and I am familiar with the above referenced patent application, as well as the development, use and properties of the anti-amyloid vaccine constructs used in the method of down-regulating amyloid described therein. Pharmexa A/S is the sole assignee of the instant patent application.

3. I have read and understood the subject matter of the Office Action of December 19, 2002 in the above-captioned patent application.

4. The following comments are offered in support of the patentability of the instant invention.

5. The instant application describes a method of down-regulating amyloid protein in a host by administering a vaccine against beta amyloid (Aβ) or amyloid precursor protein (APP) so as to treat diseases characterized excessive amyloid plaque formation.

6. In order to gauge the therapeutic potential of the method claimed in the instant application, two sets of experiments have been performed under my supervision and control.

7. In the first set, the goal has been to monitor the effect of vaccination according to the instant application against vaccination with control vaccines against the Aβ-42 molecule for treatment of Alzheimer's disease and thereby to validate the presently claimed approach to vaccination against Alzheimer's disease.

7.1 In order to reach this goal, the vaccines made according to the teachings of the instant application have been tested in 2 studies in a transgenic mouse model of Alzheimer's disease and compared to the non-modified Aβ-42 wildtype protein vaccine described by Schenk, D. et al, *Nature*, **400**; 173 – 177 (1999), cf. the attached copy thereof. The mice are transgenic for an "activated" form of the human Alzheimer's precursor protein, hAPP, and develop age related pathologies that closely resemble Alzheimer's disease characteristics. This includes amyloid plaque formation, gliosis, dystrophic neuritis and decline of cognitive functions.

7.2 In the present study the effect of vaccination on the amyloid plaque burden was examined. The presence of amyloid plaques in the brain is

generally held to be a hallmark of Alzheimer's disease and is used as a post-mortem diagnosis of said disease. To the best of my knowledge, the amyloid plaque burden is generally believed to be a correlate of the state of progression of the disease. A reduction in the amyloid plaque load in vaccinated mice is therefore likely to be a strong indication of the therapeutic effect of an anti-Alzheimer's vaccine.

7.3 The APPxPS-1 double transgenic mouse model (Holcomb, L. et al, *Nature. Med.*, **4**; 97 – 100 (1998)) was employed in the 2 studies. The mice were immunised with 50 μg of either of vaccines described in the instant application or with 100 μg control (Aβ-42) vaccine. An adjuvant only group was included as negative control. The vaccine molecules were emulsified in 100 μl of Freund's adjuvant and injected subcutaneously. A total of four to seven injections were administered, depending on the experiment, with an interval of 3 to 4 weeks.

7.4 The mice were bled 14 days after each injection and anti-Aβ-42 titres in serum were measured using standard ELISA assays.

7.5 After sacrifice, the brains were perfused and collected for analysis. Brains were sectioned and silver stained by the Switzer-Campbell method (Switzer, R.C. et al, US Patent 5192688 (1993)). The silver-stained amyloid plaques were counted and the plaque area measured by image analysis.

7.6 In the first study, five doses of vaccine were administered while seven doses were administered in the second experiment. Aβ variants as described in the instant application were freshly dissolved before emulsification with Freund's adjuvant. Aβ-42 protein was incubated for 2 – 3 days prior to use to allow fibrillation of the antigen, as described by Schenk, D. et al, *Nature*, **400**; 173 – 177 (1999).

7.7 The variants enumerated 2, 3, 13, and 17 correspond to variants 2, 3, 13, and 34, respectively, listed in the table on page 80 in the specification of the instant application. The variants enumerated 21, 22, 24, 25, 26, and 27 are all variants according to the instant application that are not explicitly disclosed,

but which all are variants falling within the scope of formula I on page 28 in the instant specification, *i.e.* they include at least one B-cell epitope of Aβ and at least one foreign T-helper epitope.

7.7 The anti-Aβ-42 titres raised by the different molecules are shown in attached Figures 1 and 2 for the two experiments, respectively.

7.8 The results reveal that several molecules according to the instant application generate significantly higher anti-Aβ titres than the control Aβ-42 wildtype molecule vaccine. In experiment 1, the Aβ-variant molecules, variant-17 and -21, produce high anti-Aβ antibody titres. In experiment 2, variant 17 (also tested in experiment 1), 24, 25, 26 and 27 all generate higher titres than the Aβ-42 control vaccine.

7.9 The silver stained plaques were counted and the plaque sizes (plaque areas) measured. Attached Figures 3, and 4 show the total plaque area in brain sections from the different mouse groups in percent of the analysed brain area. Figure 3 represents data from experiment 1 and Figure 4 shows the data from experiment 2.

7.10 These results demonstrate that three Aβ variant vaccines (variants 2, 17 and 21) in experiment 1 significantly (at a 95% confidence level) reduce the plaque burden in the brain of immunised mice, compared to the adjuvant control mice. The Aβ-42 wildtype vaccine also significantly reduces plaque load. In experiment 2, Aβ variants 17, 24, 26 and 27 significantly reduced the plaque burden, while the Aβ-42 control vaccine did not provide a statistically significant plaque reduction in this experiment.

8. Another objective has been to demonstrate that the use of the instantly claimed technology to generate anti-Aβ antibody responses eliminates or reduces the risk of generating auto-reactive T-cells specific for Aβ, as opposed to the non-modified wildtype Aβ-42 vaccine.

8.1. Auto-reactive Aβ-specific T-cells are in my opinion likely to have caused the autoimmune encephalitis-like symptoms observed in a number of patients immunised with the Aβ-42 vaccine molecule in a clinical phase II trial (Birmingham, K. & Frantz, S., *Nature. Med.* **8**; 199 – 200 (2002); Check, E., *Nature*, **415**; 462 (2002); Nicoll, J. A. R., et al, *Nature. Med.* (2003), all attached).

8.2. The basis of this expected difference between the instantly claimed vaccination approach and vaccination with the Aβ-42 vaccine molecules is the presence of very strong foreign T-helper epitopes in the vaccines used according to the instantly claimed approach; the foreign T-helper epitopes are expected to generally dominate the response. Therefore the response to endogenous and potentially dangerous Aβ-42 derived T-helper epitopes is expected to be eliminated or reduced.

8.3. The Aβ-variant vaccines and the Aβ-42 control vaccine are tested for their ability to induce Aβ-42 specific T-cells in different mouse strains by immunisation with the molecules in Freund's adjuvant as described above in paragraph 7.3. The generation of autoreactive T-cells is determined by stimulation of lymph node or spleen cells *in vitro* with Aβ-42 by measuring the degree of specific proliferation to Aβ-42 (stimulation index). The absence of proliferation (stimulation index below 2) indicates that vaccination did not lead to the generation of Aβ-specific autoreactive T-cells.

8.4 The mice are sacrificed 10-12 days after the (last) immunisation and the lymph nodes and/or spleens are taken. Single cell suspensions are prepared of lymph nodes and/or spleen in tissue culture media containing 1% syngenic mouse serum. The cells are added to tissue culture plates containing PBS and Aβ-42 protein and the cultures are incubated for 96 hours. [^{3}H]Thymidine is added to all wells for the last 20 hours of incubation and proliferation ([^{3}H]Thymidine incorporation) to PBS and Aβ-42 is measured by scintillation counting after harvesting the cells.

8.5 Aβ-42 specific proliferation was measured by *in vitro* stimulation with Aβ-42 of lymph node cells from several different mouse strains after immunisation with Aβ variant-17 or with the Aβ-42 control vaccine (cf. attached Table 1).

8.6 Aβ specific proliferation is detected when Aβ-42 is used as a vaccine, indicating the presence of Aβ-specific autoreactive T-cells in mice immunised with this vaccine. By contrast, lymph node cells from mice vaccinated with the Aβ- variant-17 does not proliferate when stimulated with Aβ-42, indicating the absence of Aβ-specific autoreactive T-cells.

8.7 In addition to variant-17, a panel of other Aβ variant molecules were tested for Aβ-42 specific proliferation, but only in the C57/Bl and Balb/c mouse strains (cf. attached Table 2). As it was observed for variant-17, none of the other tested variants (2, 13, 21, 24, 25, 26, 27) gave rise to Aβ-42 specific proliferation when lymph node cells were stimulated with Aβ-42, indicating that none of the vaccines induced Aβ-42 specific autoreactive T-cells.

9. Vaccination studies with Aβ variant vaccines using a transgenic mouse model of Alzheimer's disease revealed that several of the vaccines made and used according to the above-captioned application generated significantly higher antibody titres against Aβ-42 than the Aβ-42 vaccine itself (by up to 10 fold for variant-17). This demonstrates the capability of the modified molecules to break immunological tolerance and induce a potent immune response against a self-molecule like Aβ-42. I am of the opinion that this feature of the Aβ-variant molecules is of high importance if human patients are considered for vaccination.

10. The high titres of antibodies induced by some of the Aβ variant vaccines were shown to have some correlation with a reduced plaque burden in the transgenic mice as determined by silver staining and image analysis of brain sections from immunised mice. Six of the Aβ variant molecules (variants 2, 17, 21, 24, 26, and 27) significantly reduced the plaque load in immunised mice compared to adjuvant controls. The Aβ-42 vaccine reduced plaques in

one experiment, but not in the other. Based on these results I find that the advantage of the Aβ-variant vaccines according to the instant application also has been demonstrated at the level of therapeutic efficacy in a mouse model of Alzheimer's disease.

11. The autoimmunity experiments in my opinion demonstrate that the Aβ-variant vaccines, as opposed to the wildtype Aβ-42 control vaccine, do not activate Aβ-42 specific T-cells that could be harmful by causing autoimmune encephalitis like inflammation of the brain. This was demonstrated by the absence of Aβ-42 specific T-cell proliferation after stimulation with Aβ-42 when Aβ-variant molecules were used for vaccination. In contrast, Aβ specific proliferation was observed after Aβ-42 immunisation.

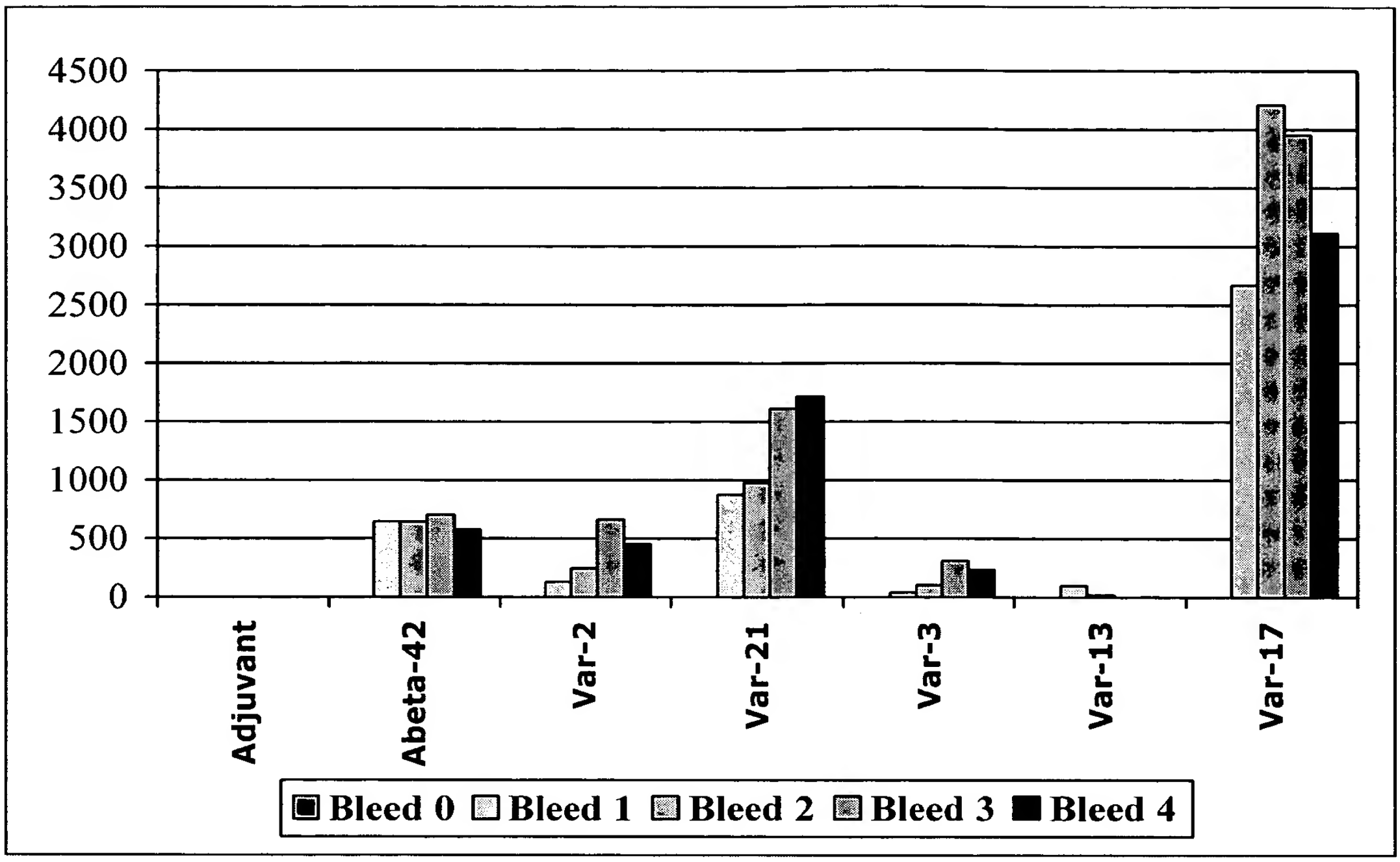
12. In conclusion, I believe that the present results show that the Aβ-variant vaccines made and used according to the instant application can generate stronger antibody responses and better therapeutic effect than the Aβ-42 vaccine and at the same time be markedly safer.

The undersigned hereby declares that all statements made herein are based upon knowledge are true, and that all statements based upon information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

DATED: May 8th, 2003

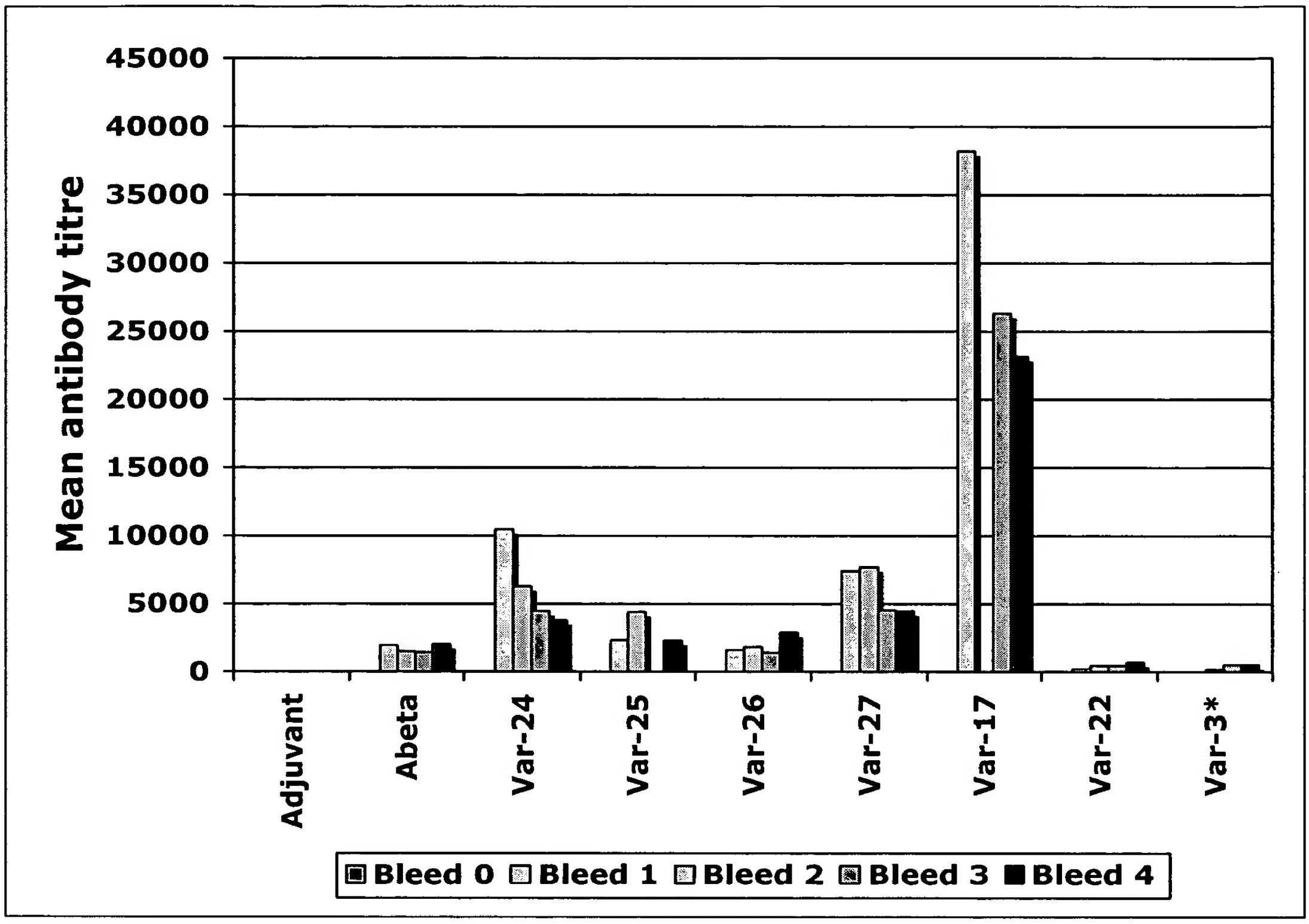
Peter Birk Rasmussen

Figure 1:



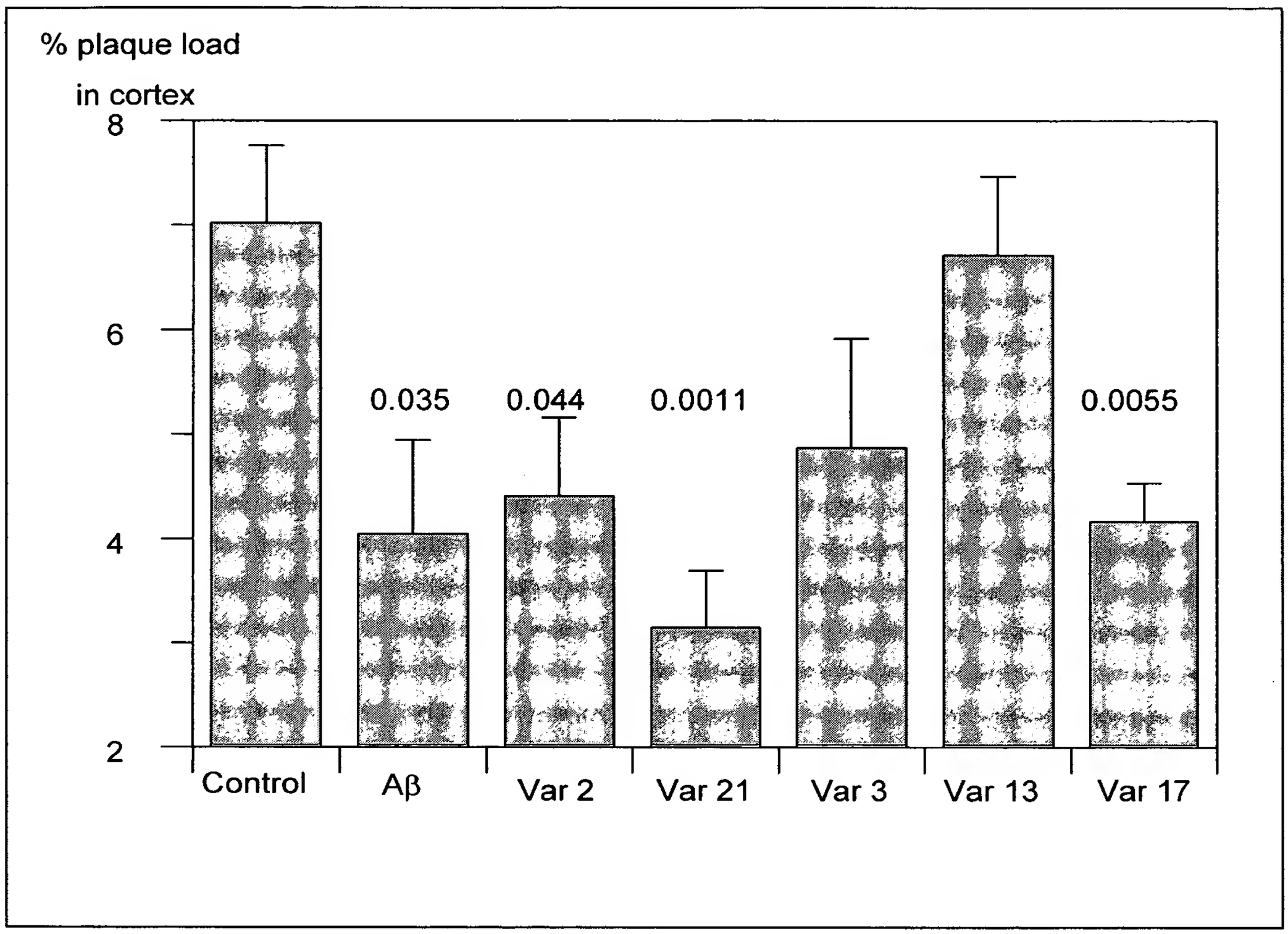
Relative Anti-Aβ-42 antibody titres in serum after vaccination of APPxPS-1 transgenic mice with Aβ variant molecules or Aβ-42 control vaccine. The adjuvant group is the negative control (adjuvant only). Titres are measured in serum from 5 bleeds corresponding to pre-bleed (bleed 0), bleed after 1st immunisation (bleed 1), bleed after 2nd immunisation (bleed 2) and so forth.

Figure 2:



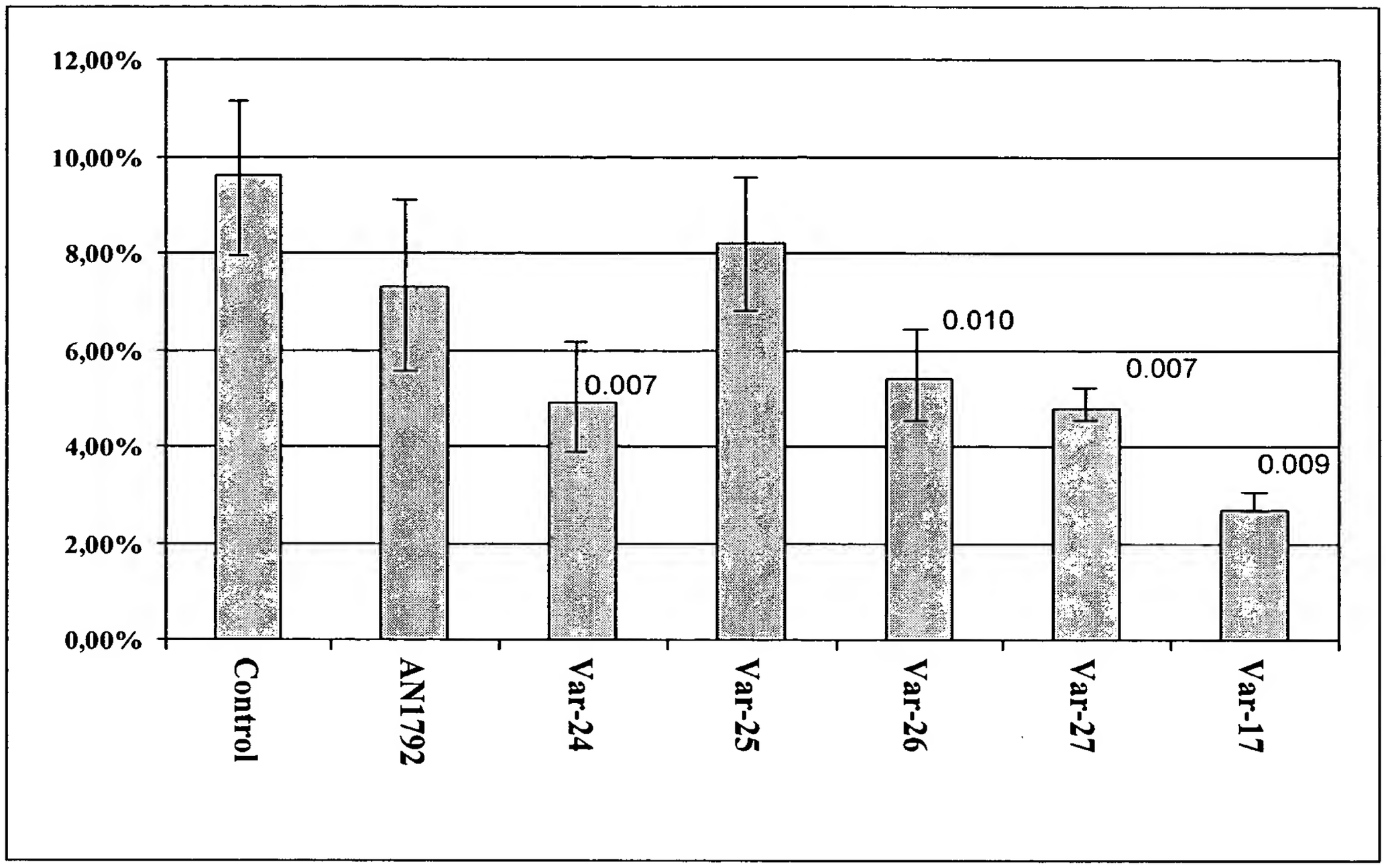
Relative Anti-Aβ-42 antibody titres in serum after vaccination of APPxPS-1 transgenic mice with Aβ variant molecules or Aβ-42 control vaccine. The adjuvant group is the negative control (adjuvant only). Titres are measured in serum from 5 bleeds corresponding to pre-bleed (bleed 0), bleed after 1st immunisation (bleed 1), bleed after 2nd immunisation (bleed 2) and so forth.

Figure 3:



Determination of plaque burden in vaccinated and control mice in experiment 1. Bars show the average plaque load in percent of the analysed brain area for each group of mice. For each mouse three different brain sections were analysed and the average plaque load of these was taken. Standard deviations for the variation between mice in each group are indicated by the thin vertical line on top of the bars. The numbers shown over some of the bars indicate the P-value (students T-test) for that group of mice compared to the control group. Control = adjuvant only.

Figure 4:



Determination of plaque burden in vaccinated and control mice in experiment 2. Bars show the average plaque load in percent of the analysed brain area for each group of mice. For each mouse four different brain sections were analysed and the average plaque load of these was taken. The thin vertical line on top of the bars indicates standard deviations for the variation between mice in each group. The numbers shown over some of the bars indicate the P-value (students T-test) for that group of mice compared to the control group. Control = adjuvant only. AN1792 = Aβ-42 vaccine.

Table 1:

Mouse strain		Stimulation index
BALB/c	Aβ-42	29
	Variant-17	1
C57/Bl	Aβ-42	5
	Variant-17	1
SJL/N	Aβ-42	10
	Variant-17	2
FVB	Aβ-42	7
	Variant-17	1
CBA/J	Aβ-42	7
	Variant-17	1

Table showing the *in vitro* proliferative responses (stimulation index) of Aβ-42 stimulated lymph node cells isolated from Aβ-42 or Aβ variant-17 immunised mice. Responses in five different strains of mice are shown.

Table 2:

Variant	Stimulation index	
	C57/Bl	BALB/c
Variant-2	1	-
Variant-13	1	-
Variant-17	1	1
Variant-21	1	10
Variant-24	1	1
Variant-25	1	1
Variant-26	1	1
Variant-27	1	1

Table showing the *in vitro* proliferative responses (stimulation index) of Aβ-42 stimulated lymph node cells isolated from C57/Bl and Balb/c mice immunised with different Aβ variants.

Curriculum Vitae, Peter Birk:

1991: Master of Science in Molecular Biology from Odense University, Denmark, on the topic "Bacterial gene regulation in *E. coli*".

1991 – 1993: E.U. Scholarship at I.N.S.A. Toulouse, France. Study on "genetic engineering of alpha-amylases".

1993 – 1995: Assistant professor at the Institute of Molecular Biology, Odense University, Denmark.

1995: Ph.D. from the Institute of Structural Chemistry at Aarhus University, Denmark.

1995 – 1997: Research Scientist at the Department of TB-Immunology at Statens Serum Institute, Copenhagen, Denmark.

1997 – : Senior Scientist and Project Manager at Pharmexa A/S, Horsholm, Denmark.